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Optical and electrical properties of bacteriorhodopsin Langmuir-Blodgett films: II. D96N mutant and its 4-keto and 9-demethyl retinal analogs

Howard H. Weetall [a,*], Anna B. Druzhko [b,1], Lynne A. Samuelson [c], Angel R. de Lera [d], Rosana Alvarez [d]

[a] *National Institute of Standards and Technology, Biotechnology Division, Gaithersburg, MD 20899-0001, USA*
[b] *National Institute of Standards and Technology, Gaithersburg, MD 20899-0001, USA*
[c] *US Army Natick Research, Development and Engineering Center, Biotechnology Division, Natick, MA 01760-5020, USA*
[d] *Departamento de Quimica Pura y Aplicada, Facultad de Ciencias, University de Virgo, Virgo 36200, Spain*

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Abstract

Langmuir-Blodgett films prepared from purple membranes of the D96N mutant and two of its chromophore analogs (4-keto-retinal and 9-demethyl-retinal) were characterized for M-state kinetics and photocurrent production. These studies indicated that unlike the wild-type (WT) and its identical chromophore analogs, the photocurrent transients were unusual, showing proton release at pH 3.0. Several possible explanations for these results are considered. © 1997 Elsevier Science S.A.

Keywords: Langmuir-Blodgett films; Bacteriorhodopsin; Purple membranes; Photocurrents

1. Introduction

Bacteriorhodopsin (bR) is a light-sensitive transmembrane protein produced by the bacterium *Halobacterium salinarium*. This transmembrane protein is located in a structurally distinct area of the plasma membrane known as the 'purple membrane' (PM) [1]. When activated by yellow light, it undergoes a photocycle whereby a proton is transported from the cytoplasmic side of the membrane to the periplasmic space. The yellow light induces a *trans–cis* isomerization of the chromophore (retinal) bound to Lys-216. This isomerization is followed by a series of transitions of the protein–retinal complex through a number of intermediates (I, J, K, L, M, N, and O) that are characterized by different protonation states of the retinal Schiff base and of several amino acid residues within the protein [2,3]. The longest lasting intermediate state is the M-state. In passing from the ground state to the M-state, the bR releases a proton to the periplasmic space. During the decay of the M-state back to ground state, the Schiff base is reprotonated.

In a previous report [4], we described the release and uptake of protons by Langmuir-Blodgett (L-B) films of the wild-type (WT) bR. These studies were carried out on antimony tin oxide (ATO) electrodes in a flow-through electrochemical cell [4–6]. We have now examined the L-B films of the D96N mutant using the same approach. In the D96N mutant, the Asp at position 96 is replaced by Asn. In addition, we have, for the first time, examined the proton uptake and release characteristics of two retinal analogs of the WT and the D96N; 4-keto-retinal and 9-demethyl-retinal. The 4-keto analog has a modification on the retinal ring, while the 9-demethyl-retinal has a modification on the polyene chain. We have also examined the S35C mutant. This mutant has the Ser-35 replaced by Cys. This mutation is external to the membrane and is therefore exposed to the external environment.

2. Materials and methods

2.1. Preparation of the bR analogs

Apomembranes were prepared by the standard protocol [7]. An aqueous suspension of WT and D96N mutant were

* Corresponding author.
[1] Present address. Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region, Russian Federation.

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illuminated with intense light in the presence of 0.5 M NH_2OH, at pH 8.2 to 8.4 at 7°C. The formation of the apomembranes was followed by the decrease in optical density at 400 nm. For reconstruction, the analogs were first dissolved in isopropanol before adding to the apomembranes in aqueous suspension. The 9-demethyl-retinal bR derivative was prepared as previously described [8].

2.2. Preparation of Langmuir-Blodgett films

The L-B films were prepared as previously described [9] by suspending the purple membrane fragments containing bR in reagent grade hexane. The films were prepared on a Lauda MGW Filmwaag Balance. MilliQ deionized water was used as the subphase and maintained at 30°C. The bR spreading solution was prepared by adding approximately 1 mg of bR (from a 4 mg/ml to 5 mg/ml water stock solution) to 2 ml of hexane. The mixture was sonicated for 1 min before spreading. The bR was then allowed to sit for approximately 10 min on the water surface before compression. Films were compressed to an annealing target pressure of 20 mN/m. In all cases, the films were left to anneal for at least 20 min until constant pressure was maintained. The ATO electrodes were pretreated with Malinkrodt Chem-Solv (Malinkrodt Chemicals, Paris, Kentucky), rinsed with water and passed once through the monolayer, removed and allowed to air dry.

2.3. Electrochemical setup

The electrode setup has been previously described [5,10]. It consisted of a flow-cell prepared from a commercially available electrochemical cell (BAS CC-4 Flow-Cell) modified to hold the glass-coated ATO electrode and having a black nylon cell cover with a 5-mm diameter hole directly over the L-B film for irradiation. The metal body of the cell served as the counter electrode and a silver/silver chloride electrode as reference. The spot under the hole was illuminated with a mercury-arc lamp (LEP, HBO 100). The cell was connected to a potentiosat having an operational amplifier with a 1-MΩ resistor in a feedback circuit. The entire cell was shielded by placement in an aluminum box. The system was connected to an oscilloscope capable of giving a response time of approximately 4 ms. The polarity of the signal was adjusted so that a pH decrease caused an increase in the observed current transient, while increased pH produced a decrease in the observed current transient. It is well-known that tin oxide, when exposed to DC current for a long period of time, changes in character. In our system, the tin oxide electrodes used for each experiment were new. The background current produced by these electrodes, at the applied voltages, were small, and therefore had little effect on the characteristics of the tin oxide electrode through the course of the experiment. Therefore, at least for a first approximation, we assumed that direct monitoring of the actual current and voltage was unnecessary, since we were only interested in the current transient produced by the addition and removal of the light. The photocurrent transients were determined at pH 7.0 and 3.0 in 0.005 M phosphate buffer, 0.05 M KCl. The light-on transient was observed immediately upon irradiation. The samples were irradiated with yellow light for 1 s before the light was shut off by a computer-operated electronic shutter. Each value for the observed photocurrent transient is the average of 5 separate determinations on the same electrode. The relative uncertainty of the currents observed between different electrodes is approximately 14%, and in most cases did not exceed the standard deviations.

2.4. Spectroscopy

Spectroscopy was carried out on a diode array spectrophotometer (HP-8452A). The films were irradiated with 25 mW/cm^2 of yellow light from a 300-W halogen Kodak projector lamp through a 530-nm long-pass filter (Oriel 59500), so that no 560-nm light passed the filter. The L-B

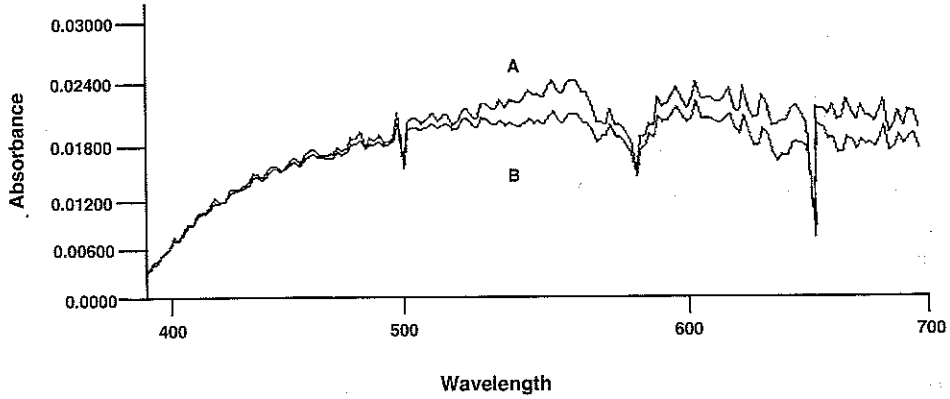

Fig. 1. Spectra of the D96N mutant L-B film with the light off (A) and the light on (B). The films were exposed to yellow light with a filter that cut off all light below $\lambda = 530$ nm.

Table 1
Summary of photocurrent transients

bR type	Mean peak current (nA)+standard deviation	
	pH 7.0	pH 3.0
WT	13.7±1.6	−7.0±1.0
9-demethyl-WT	1.0±1.0	8.0±2.0
4-keto-WT	2.2±1.0	−0.8±1.0
D96N	6.5±1.0	NA
9-demethyl-D96N	4.2±1.0	8.0±1.0
4-keto-D96N	2.0±1.0	NA
S35C	20 ±2.1	−11.0±2.0

The term NA denotes our inability to obtain usable data.
Each value represents an average of 5 determinations on a single ATO electrode.

films were examined spectrophotometrically in the dry state. Where possible, M-state recovery times were determined, and M-state decay half-times ($T_{1/2}$) calculated using a single exponential model.

We were unable to obtain data from several of the samples because the noise levels or the M-state recovery times were faster that the capabilities of our instrumentation. In all cases, the half-times were calculated from the data collected at 560 nm, in the dark, over a 30-s to 60-s period after exposure to the light source.

3. Results

The L-B films all showed slight differences between the light-on and the light-off spectra (Fig. 1). However, none of the L-B samples showed a well-defined peak. Although the spectral data were noisy, differences were observable over the spectral range from approximately 500 to 700 nm. We were unable to obtain a sharp distinct spectral maxima from all the samples examined. The wavelength of 560 nm was chosen for the kinetic studies because in all the samples tested, this appeared to be where the largest spectral difference was observed between the light-on and light-off conditions. The half-times for the M-state decay ranged from 0.1 to 0.6 s. Operation of the spectrophotometer at a cycle time of 100 ms/cycle with a 100 ms integration time (fastest cycle and integration times on this system) could not distinguish statistically significant kinetic differences. The data were finally collected using 0.5 s cycle time and a 0.2-s integration time. Although we could calculate a M-state decay time, the reproducibility was exceptionally poor. These values varied by as much as 100% on subsequent trials.

The photocurrent transient data, summarized in Table 1, yielded some unexpected results. The photocurrent transients previously produced from unoriented films of the WT and the D96N mutant showed similar proton release characteristics on irradiation [5,6,10]. The photocurrent transients observed on elimination of the yellow light were much slower in the case of the D96N mutant than with the WT [6]. The results observed with D96N mutant in the L-B films were different than in the unoriented non-L-B films. The photocurrent-transient of the WT, as expected, showed rapid proton release and uptake. The D96N on the other hand, well known for having a slower M-state recovery time, showed a similar proton release current transient at pH 7.0 (Fig. 2). However, at pH 3.0 where a reversal in the direction of the light-on transient is observed in the unoriented films, we observed a proton release transient that increased slowly over the 1 s light exposure. Elimination of the yellow light brought the observed current slowly back to baseline with no transient proton uptake peak (Fig. 3). The analogs of the D96N mutant showed photocurrent transients similar to the D96N mutant L-B film (Figs. 4 and 5). In both the 4-keto-retinal and the

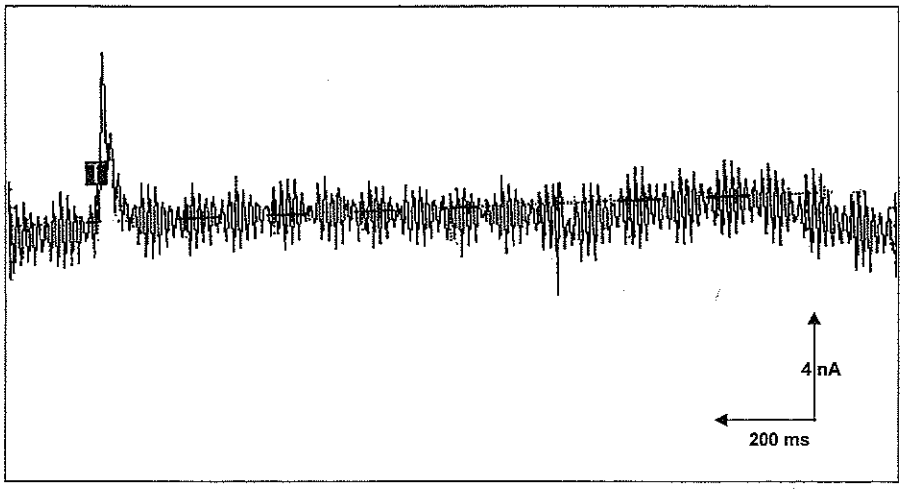

Fig. 2. Photocurrent transient of the D96N mutant at pH 7.0. Note that at time T (light-on), there is an immediate rise in current representing a decrease in pH (proton release). Although the proton uptake transient is within the noise, there appears to be a slight change immediately after removal of the yellow light.

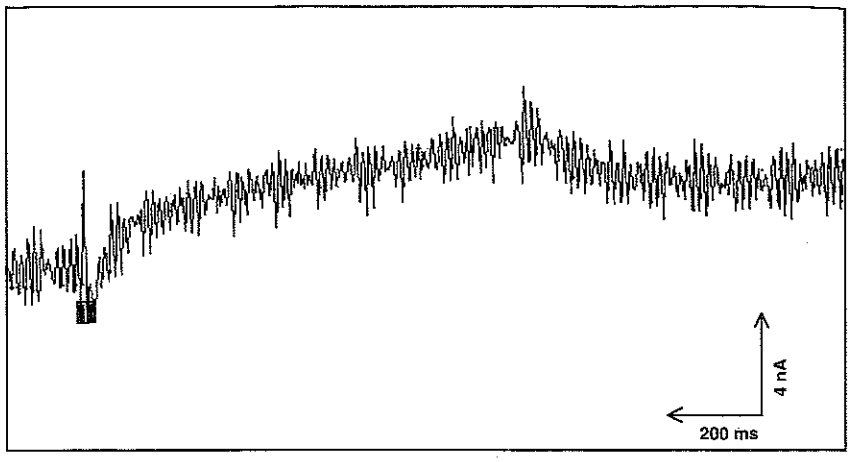

Fig. 3. Photocurrent transient of the D96N mutant at pH 3.0. Note that at time T (light-on), the current slowly rises (proton release), reaching a maximum and moving toward baseline when the light is removed. There is no observable proton uptake.

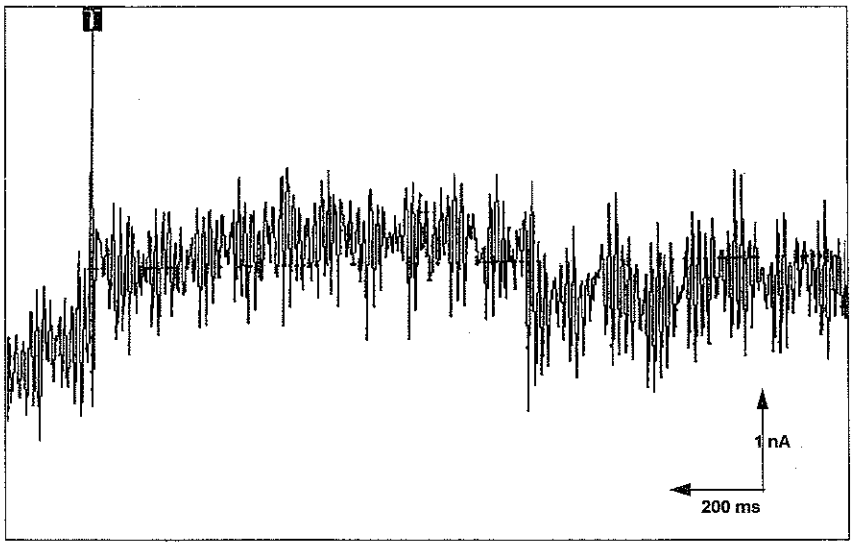

Fig. 4. Photocurrent transient of the D96N mutant 4-keto-retinal analog at pH 3.0. Note that at time T (light-on) the current rises to what appears to be a steady-state and immediately returns to baseline when the light is removed. No proton uptake is observed.

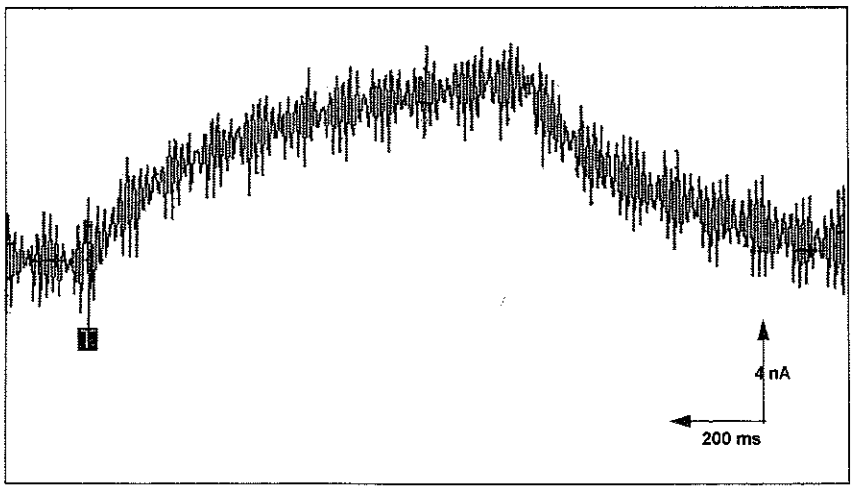

Fig. 5. Photocurrent transient of the D96N mutant 9-demethyl-retinal analog at pH 3.0. Note that at time T (light-on), there is a slow rise in current (proton release), approaching what appears to be a steady-state. On removal of the yellow light, the current slowly returns to baseline. There is no apparent proton uptake.

9-demethyl-retinal derivatives, proton release was observed with no detectable uptake. However, the 4-keto derivative showed a more rapid rise on irradiation and similarly a more rapid decrease in current on removal of the yellow light. The 9-demethyl derivative appeared to act more like the D96N containing the native chromophore. When irradiated with yellow light, there was a slow rise in current (proton release) that appears to move toward a steady state. When the light was turned off after 1 s of exposure, the photocurrent signal slowly returned to ground state. None of the D96N samples showed proton uptake. The S35C mutant showed a photocurrent transient similar to the previously published WT transient (Fig. 6) [5].

4. Discussion

The preparation of the apomembranes and the process of reconstruction have been discussed in a previous publication [5]. The removal of the retinal oxime from the bR was found to be unnecessary, since the observed photoelectrical results with and without the presence of the retinal oxime were identical [5]. All the studies reported in this paper were with analogs prepared by photolysis and containing the retinal oxime in the reconstructed analogs.

The spectra of the bR containing L-B films in the light and the dark did indicate optical activity and an active photocycle. The optoelectrical data confirms the biological activity of the L-B films.

In our previous reports, using unoriented bR films at pH 7.0 on ATO [5,6,11], we observed similar type responses from WT, D96N, D96E and S35C. The differences were not in the direction of the light-on/light-off transients, but in the shape and half-times of the light-off photocurrent transients. These differences in the light-off photocurrent transients were similar to the M-state decay differences observed spectrally in gelatin films [6,11,12], where the water content was decreased sufficiently to effect the M-state decay kinetics. This phenomenon has been studied in some detail [13,14]. It was concluded that dehydration-induced changes in kinetic parameters of both WT and D96N bR gelatin films result from different pathways of the Schiff base reprotonation. The major reason that the M-state lifetimes in the D96N bR films are largely unaffected by humidity, is the lack of the proton translocation step from Asp-96 to the Schiff base. This step is humidity-dependent in the WT-bR [14].

Our previous data on transient photocurrents generated from unoriented bR ATO films (4-keto-retinal and the 9-demethyl-retinal analog derivatives of bR reconstructed into the WT bR)[5] showed similar type results, in that the lifetimes of the WT bR changed more dramatically with the addition of the analog than in the case of the D96N bR L-B films. These differences were most noticeable in the pH profiles of the bR WT and D96N analog derivatives as compared to the bR ATO films containing native chromophore. The pH at which the light-on phototransient reversed from proton release to proton uptake, and the extent of the reversal was most striking in the WT analogs as compared to the D96N analogs. In a recent study of WT oriented L-B films on ATO [4], we observed results similar to those seen with the unoriented films prepared on ATO [5]. The L-B films are between 65 to 95% oriented when prepared [15,16].

In summary, previous spectral observations on gelatin films [6,11,12,14], photocurrent transient data on ATO unoriented films of WT, and D96N mutant [5,6,11] and photocurrent transient data on WT-bR and D96N analogs [5,12,14,17] indicate that the WT is most affected by dehydration (formation of the film), while the D96N is only slightly affected. Examination of the oriented WT bR in L-B films [4] gave results similar to the unoriented films previously examined on ATO [5]. This does not appear to be the case for the oriented L-B films of the D96N mutant on ATO. The pH 7.0 light-on photocurrent transient (Fig. 2) looks similar to transients observed in previous studies,

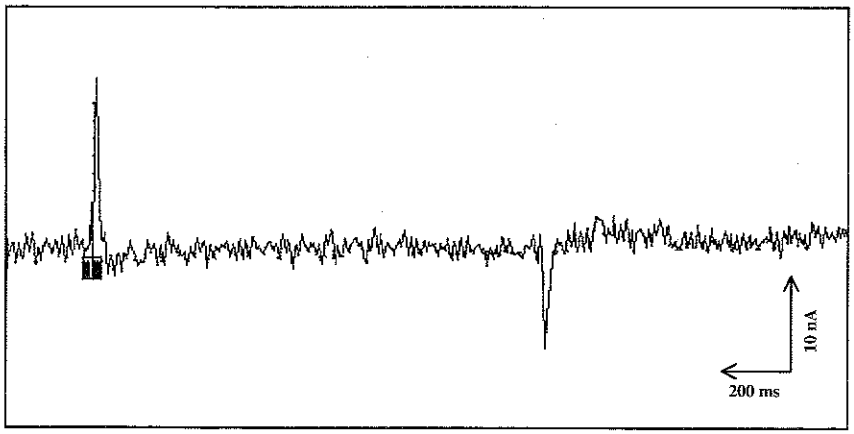

Fig. 6. Photocurrent transient of the S35C mutant containing the native chromophore at pH 7.0. At time T (light-on) there is an immediate transient current (proton release). When the yellow light is removed, there is an immediate reverse transient (proton uptake). This figure is identical to the previously published photocurrent transient for L-B WT bR.

showing rapid proton release. However, the pH 3.0 photocurrent transient is very different (Fig. 3). In all previous examples of the D96N mutant containing the native chromophore or either the 4-keto or the 9-demethyl-retinal at pH 3.0, the light-on photocurrent transient indicated proton uptake (it was reversed from pH 7.0). The D96N bR in the oriented L-B film containing the native retinal or either of the analogs does not show a reversal of proton release (proton-uptake) on irradiation, but shows very slow proton release (Figs. 3–5) over the entire 1-s light-on episode. The rate of proton release appears to approach a constant turnover. Upon removal of the yellow light, the rate of proton release decreases back toward the baseline. Unlike the previously examined bR samples, the D96N bR L-B films show no observable uptake.

Earlier reports on bR-L-B films [15,16] claim that the protein is oriented cytoplasmic side toward the electrode surface. They also claim that they observe charge separation. Purple membranes of WT bR oriented on tin oxide electrodes by use of antibodies specific to epitopes on the cytoplasmic and external sides of the molecule [16] produce transient photocurrents, at around pH 7–8, that always show a pH decrease, although the size of the signal varies considerably between the two. It has been claimed that the protons move toward the cytoplasmic side [18]. It has also been suggested that a rectified photocurrent exists when the bR orientation is random [16]. They explain this by suggesting that an electrochemical interfacial effect externally controls, through an electrostatic field developed in the double layer, the efficiency of charge displacement through the suppression of charge displacements in the cathodic direction. The trace response observed in the inversely oriented sample was because few cathodically responsive fragments of the bR remain.

The slow movement of protons to the surface of the electrode in our D96N L-B films, at acid pH, suggests that in the case of the D96N mutant, the orientation may be external side up. In the case of the D96N mutant, the light-on transient, in our hands, has always been observed to be rapid. In addition, our previous data on unoriented films of D96N indicate that at pH 3.0, the proton release is greatly slowed or stopped. In this study, we observe proton release at acid pH. Although the proton release was very slow, the photocurrent response is similar in shape to previously observed proton uptake photocurrent responses in the D96N mutant at the same pH. If the cytoplasmic side of the bR membrane fragments are facing the electrode surface, and if the protons are being released slowly from the cytoplasmic side, toward the electrode surface, as has been suggested [15,16,18], this could explain our observations. However, it has been suggested [16], that there may be a few remaining PMs oriented in the opposite direction. At acid pH, most of the bR proton release is suppressed, yet a small amount could still release protons or show charge displacement in the cathodic direction. However, our system is not sufficiently sensitive to observe charge displacement. It must be remembered that all the previous work on photocurrent production utilized indium tin oxide electrodes (ITO). We have observed that ITO, when exposed to yellow light shows a small photocurrent. The ATO, however shows absolutely no response to yellow light. There is a possibility that previously collected data and their interpretation [15,16], and our data and its interpretation is related to the light-induced photocurrent by the ITO electrodes. It is also possible that the D96N mutant and its analogs in the L-B films are cytoplasmic side up, and the observed slow release is actually caused by the dehydration of the film, slowing down the deprotonation step, as well as the reprotonation step. After dehydration to approximately 12% humidity, the kinetics of the D96N mutant M-state decay are slowed by approximately a factor of four [6,14]. Similarly, the photopotentials of the D96N ATO films are also affected by decreasing the humidity [17]. The photopotential decreases with decreasing humidity from a maximum at roughly 50% humidity by a factor of 4 at around 12% [17]. The decreased humidity also effects the photocurrent [17] by decreasing its size and shape. The ATO films used in the humidity study were electrophoretically oriented external side down. The shape of these curves did not exactly mimic ours. However, the overall direction of the observations were similar. It is possible that the data observed with the D96N L-B films are due to poor hydration as compared to the WT bR L-B films and to some degree of random orientation. We cannot eliminate the possibility that the change from the photoactive ITO to ATO had an effect on the observed photocurrents, and therefore had an influence on the interpretation of our data versus previously published results [15,16].

The S35C mutant L-B film photocurrent transient at pH 7.0 (Fig. 6) and at pH 3.0 (not shown) are identical to the WT L-B films previously studied [4]. The transient photocurrents generated by this mutant are also similar to those observed with WT bR in unoriented films [5]. This would tend to suggest that dehydration, at least, with this mutant is not affecting its properties. This lends more credence to the possibility that the L-B film is hydrophobic side down, at least, after being placed in an aqueous environment.

At the present time we are unable to explain the unique differences in observed pH 3.0 photocurrents with the D96N mutant L-B films and its analogs.

5. Conclusions

Dramatic differences were observed in the photocurrent transients produced by the D96N mutant bR in an L-B film at pH 3.0. The 4-keto and the 9-demethyl retinal analogs of this mutant gave results similar to the same mutant bR containing the native chromophore. The major differences observed in the D96N films, at pH 3.0, was an elongated

proton release signal that appeared to reach a steady state within the 1 s of exposed yellow light instead of the expected rapid light-on transient proton uptake signal. Removal of the yellow light caused the proton release rate to decrease toward the baseline. No light-off proton uptake signal was observed in any of the films examined at pH 3.0. We suggest three possible explanations for this observation. The first possibility is consistent with the suggestion that the bR is cytoplasmic side down on the ATO electrode, and that we are observing a suppression of charge displacement in the cathodic direction with the signal coming from the remaining cathodic responsive purple membrane fragments releasing protons or causing a charge displacement in the cathodic direction [16]. The second explanation is related to the effect of humidity on the bR in the L-B film [14,17]. Decreased humidity can cause large changes in the size and duration of the transient light-on photocurrent. Observed decreases in photocurrents caused by lowering humidity can range from four- to twentyfold. The third possibility is that the signal is due the photoactivity of the ITO electrodes versus our ATO electrodes. The ITO, in our hands, shows a photocurrent in yellow light, while the ATO shows no response under identical conditions. At this time, we cannot distinguish between these three alternatives. It is possible that none or all of these explanations are relevant to the results observed with the D96N mutant bR oriented L-B films and its analogs.

We plan to continue examining this phenomenon and believe that we will soon be able to provide a more detailed perspective after further experimentation.

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